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Investigation of SNPs in the *ATP1A2*, *CA3* and *DECR1* genes mapped to porcine chromosome 4: analysis in groups of pigs divergent for meat production and quality traits

Roberta Davoli¹, Luca Fontanesi¹, Silvia Braglia¹, Irma Nisi¹,
Emilio Scotti¹, Luca Buttazzoni², Vincenzo Russo¹

¹Dipartimento di Protezione e Valorizzazione Agroalimentare. Università di Bologna, Italy

²Associazione Nazionale Allevatori Suini. Roma, Italy

Corresponding author: Prof. Roberta Davoli. DIPROVAL, Sezione di Allevamenti Zootecnici. Università di Bologna. Via F.lli Rosselli 107, 42100 Coviolo, Reggio Emilia, Italy - Tel. +39 0522 290507 - Fax: +39 0522 290523
Email: roberta.davoli@unibo.it

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ABSTRACT

Three genes (ATPase, Na⁺/K⁺ transporting, α 2(+) polypeptide, *ATP1A2*; carbonic anhydrase III, *CA3*; 2,4-dienoyl CoA reductase 1, mitochondrial, *DECR1*), isolated from a porcine skeletal muscle cDNA library and mapped on porcine chromosome 4 (SSC4), were investigated. A new single nucleotide polymorphism (SNP) was identified in the 3'-untranslated region of the *CA3* gene and used to genetically map this locus on SSC4 together with the *ATP1A2* and *DECR1* loci for which SNPs were already reported. Allele frequencies of the three loci were reported for 11 pig breeds (Italian Large White, Italian Landrace, Italian Duroc, Belgian Landrace, Hampshire, Piétrain, Meishan, Cinta Senese, Casertana, Calabrese and Nero Siciliano). Radiation hybrid mapping of these genes confirmed the linkage mapping results as well as mapping information reported by other authors. Then, the SNPs identified in the *ATP1A2*, *CA3* and *DECR1* genes were genotyped in Italian Large White and Italian Duroc animal groups with extreme and divergent estimated breeding value for several production traits. For *CA3* significant differences in allele frequencies ($P < 0.05$) were observed between the extreme groups of pigs for the lean cuts (Italian Large White) and visible intermuscular fat (Italian Duroc) traits. For *DECR1*, a significant difference in allele frequencies was observed only for the visible intermuscular fat trait. *ATP1A2*, which maps close to the *FAT1* locus, did not show any significant difference. A very high linkage disequilibrium ($D' = 0.967$; $P < 0.0001$) was identified between *CA3* and *DECR1* in the Italian Duroc population. Further investigations are needed to evaluate the effect of *CA3* and *DECR1* on the considered traits.

Key words: *ATP1A2*, *CA3*, *DECR1*, Meat production traits, Pig chromosome 4.

RIASSUNTO

STUDIO DI TRE GENI (*ATP1A2*, *CA3* E *DECR1*) LOCALIZZATI SUL CROMOSOMA SUINO 4: ANALISI DELLE FREQUENZE ALLELICHE DI SNP IN SUINI ESTREMI PER ALCUNI CARATTERI PRODUTTIVI

Tre geni (ATPase, Na⁺/K⁺ transporting, α 2(+) polipeptide, ATP1A2; carbonic anhydrase III, CA3; 2,4-dienoyl CoA reductase 1, mitocondriale, DECR1), isolati da una libreria a cDNA ottenuta da muscolo scheletrico di suino, sono stati scelti per questo studio sulla base del ruolo fisiologico della proteina codificata in processi cellulari e metabolici collegabili in modo diretto o indiretto con alcuni caratteri produttivi. La scelta dei

tre geni è stata anche basata sulla loro localizzazione sul cromosoma 4 dove diversi QTL per caratteri produttivi sono già stati identificati.

Nella regione 3' non tradotta del gene CA3 è stato identificato, mediante analisi SSCP, un polimorfismo biallelico. Il sequenziamento dei due alleli ha permesso di identificare una nuova mutazione puntiforme, che è stata successivamente analizzata mediante PCR-RFLP. Per questo gene è stato effettuato il mappaggio genetico sul cromosoma 4 mediante l'analisi della mutazione nei campioni delle famiglie di riferimento del progetto europeo di mappaggio del genoma suino (PiGMap). Utilizzando il polimorfismo PCR-RFLP del gene ATP1A2, già descritto in un precedente lavoro come SSCP, sono state tipizzate diverse famiglie di riferimento PiGMap ed è stato confermato il mappaggio genetico di ATP1A2. Utilizzando queste informazioni e quelle già disponibili per DECR1, per la prima volta, è stata ottenuta una mappa di linkage del cromosoma 4 che comprende tutti e tre i geni analizzati. Il mappaggio genetico dei tre geni è stato anche confermato mediante tipizzazione di un pannello di ibridi di cellule irradiate (IMpRH 7000 rad).

Le frequenze alleliche delle mutazioni identificate nei tre loci sono state studiate in 11 razze suine (Large White Italiana, Landrace Italiana, Duroc Italiana, Landrace Belga, Hampshire, Piétrain, Meishan, Cinta Senese, Casertana, Calabrese and Nero Siciliano) per un totale di 272 animali. Inoltre, come approccio iniziale per poi scegliere i geni da analizzare in futuri studi di associazione, abbiamo confrontato le frequenze alleliche di mutazioni puntiformi per questi tre loci in gruppi di suini di razza Large White Italiana e Duroc Italiana, analizzando animali con valori degli indici genetici estremi e divergenti per alcuni caratteri produttivi (accrescimento, spessore lardo dorsale, tagli magri e grasso intermuscolare visibile). Per il gene CA3 è stata osservata una differenza nella distribuzione delle frequenze alleliche ($P < 0,05$) per i due caratteri tagli magri (nella razza Large White Italiana) e grasso intermuscolare visibile (nella razza Duroc Italiana). Per il gene DECR1, differenze significative sono state osservate per il carattere grasso intermuscolare visibile. Il gene ATP1A2, che mappa vicino al locus FAT1, non ha presentato differenze statisticamente significative nelle frequenze tra i gruppi estremi per i caratteri oggetto di studio. Analizzando il livello di linkage disequilibrium (LD) tra i tre loci, è stato evidenziato un elevato livello di LD ($D' = 0,967$; $P < 0,0001$) tra i geni CA3 e DECR1, solo nella popolazione Duroc Italiana. Questi primi risultati pongono le basi per ulteriori studi per verificare se i geni CA3 e DECR1 sono associati con i caratteri oggetto di selezione nel suino pesante.

Parole chiave: ATP1A2, CA3, DECR1, Qualità della carcassa e della carne, Cromosoma 4 di suino.

Introduction

Several studies involving crosses between different breeds or lines (i.e.: Andersson *et al.*, 1994; Walling *et al.*, 1998; Wang *et al.*, 1998; Perez-Enciso *et al.*, 2000; Bidanel *et al.*, 2001; Cepica *et al.*, 2003) have indicated that porcine chromosome 4 (SSC4) harbours quantitative trait loci (QTL) affecting growth, fat deposition, fat composition and carcass traits. Segregation of QTL on this chromosome has also been observed in commercial pig populations (Evans *et al.*, 2003; Nagamine *et al.*, 2003) and attempts to fine map the QTL have been carried out with the final objective to identify the causative mutation(s) (Walling *et al.*, 2000; Moller *et al.*, 2004).

With the aim to identify candidate genes for meat production traits, we described the isolation of more than 1000 expressed sequence tags (ESTs) from a porcine skeletal

muscle cDNA library and mapped some genes to SSC4 (Davoli *et al.*, 1999, 2002a, 2002b; Fontanesi *et al.*, 1999). Three of them, ATPase, Na^+/K^+ transporting, α 2(+) polypeptide (ATP1A2), carbonic anhydrase III (CA3) and 2,4-dienoyl CoA reductase 1, mitochondrial (DECR1), have been selected in order to identify DNA markers and to evaluate the distribution of allele frequencies in pigs divergent for meat quality and production traits. These genes were chosen considering their functions and their map localizations (SSC4q21-q23, SSCq11-q16 and SSCq15-q16, respectively) close to the QTL already reported.

ATP1A2 is highly expressed in skeletal muscle and neural tissues. Variation in this gene has been associated in humans with resting relative fuel oxidation rates (Katzmarzyk *et al.*, 1999), increase in total fat mass, low-density lipoprotein cholesterol and skeletal muscle glycolytic-to-oxidative

enzyme ratio (Ukkola *et al.*, 2003). In a previous study (Russo *et al.*, 1999), in the 3'-untranslated region (3'-UTR) of the porcine *ATPIA2* gene, we observed a single strand conformation polymorphism (SSCP) and then identified the G>C causing point mutation (position 28 of sequences AM040712 and AM040711). This SSCP was utilised to genetically map this locus on SSC4 (Russo *et al.*, 1999; Blazková *et al.*, 2000).

CA3 has been identified mainly in skeletal muscle and adipocytes in which represent up to 25-30% of the soluble proteins (Lynch *et al.*, 1993; Wetzel and Gross, 2000) and seems to be involved in the protection of cells from oxidative damage (Raisanen *et al.*, 1999). In rats, an obesity related-decrease in both the concentration and activity of CA3 was observed in lipogenic tissues (Lynch *et al.*, 1993).

DECR1 is involved in an alternative pathway of β -oxidation of polyunsaturated enoyl-CoAs. In humans, a reduced activity of this enzyme was associated with an unusual accumulation of 2-*trans*,4-*cis*-decadienoylcarnitine in urine and blood derived from incomplete oxidation of linoleic acid (Roe *et al.*, 1990). We have identified a missense mutation in exon 2 of the porcine *DECR1* gene (V54L) in a region that is highly conserved in mammals and chickens, just upstream from the putative NADP⁺ binding site of the protein. This polymorphism was used to linkage map this gene in the PiGMap resource populations (Davoli *et al.*, 2002a).

Here we describe the identification of a new single nucleotide polymorphism (SNP) in the porcine *CA3* gene and the construction of genetic and radiation hybrid maps of SSC4 including, for the first time, all three of these genes. Then, in order to select genes to be investigated in more detail in association studies with production traits, as a first approach, the SNPs identified or already described in the *ATPIA2*, *CA3* and *DECR1* genes were analysed to compare their allele frequencies in groups of sib tested pigs (Italian Large White and Italian Duroc) with

extreme estimated breeding values (EBV) for several production traits.

Material and methods

Polymerase chain reaction (PCR), identification and analysis of mutations

CA3: PCR primers (forward: 5'-ATTTCTGGCTTATGTGTAAT-3'; reverse: 5'-CCATTTTGTGTCATCTTA-3'), that amplify a fragment of 148 bp, were designed on sequence AJ301338 (Davoli *et al.*, 2002b) which corresponds to a portion of the 3'-UTR of the porcine *CA3* gene (BLASTN e-value = 3e-19 against the human *CA3* cDNA 3'-UTR sequence included in NM_005181). PCR was performed on a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) in a 20- μ l reaction containing 50-100 ng porcine genomic DNA, 10 pmol per primer, 250 mM each dNTP, 1.5 mM MgCl₂ and 1 U EuroTaq DNA polymerase (EuroClone Ltd., Paignton, Devon, UK). PCR profile was as follows: an initial denaturation step (5 min at 95 °C) followed by 35 cycles (30 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C) and a final extension step (5 min at 72 °C). The resulting amplicon, initially obtained from genomic DNA of 19 pigs of different breeds (3 Large White, 3 Landrace, 3 Duroc, 3 Pietrain, 2 Belgian Landrace, 3 Hampshire and 2 Meishan), was analysed to search for SSCPs as previously described (Fontanesi *et al.*, 2001). For the sequencing of the PCR fragments, 3-4 μ l of the PCR product was treated with 2 μ l of ExoSAP-IT[®] (USB Corporation, Cleveland, Ohio, USA) following the manufacturer protocol. Sequencing reactions were carried out using the BigDye v. 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the same PCR primers, followed with a purification step with DyeEx[™] 2.0 Spin columns (Qiagen, Hilden, Germany). Sequencing products were loaded in an ABI3100 Avant (Applied Biosystems) sequencer and sequence data were analysed with Sequencing Analysis 3.7 software (Applied Biosystems). The mutation at the

Table 1. Allele frequencies at the *ATP1A2*, *CA3* and *DECRI* loci.

| Breeds | <i>ATP1A2</i> | | | <i>CA3</i> | | | <i>DECRI</i> | | |
|---------------------|---------------|----------|----------|------------|----------|----------|--------------|----------|----------|
| | N. of pigs | Allele C | Allele G | N. of pigs | Allele C | Allele T | N. of pigs | Allele G | Allele C |
| Italian Large White | 57 | 0.272 | 0.728 | 46 | 0.152 | 0.848 | 68 | 0.397 | 0.603 |
| Italian Landrace | 25 | 0.760 | 0.240 | 56 | 0.732 | 0.268 | 45 | 0.622 | 0.378 |
| Italian Duroc | 31 | 0.500 | 0.500 | 46 | 0.707 | 0.293 | 56 | 0.661 | 0.339 |
| Belgian Landrace | 17 | 0.618 | 0.382 | 18 | 0.472 | 0.528 | 17 | 0.440 | 0.560 |
| Hampshire | 20 | 0.100 | 0.900 | 16 | 0.469 | 0.531 | 18 | 0.360 | 0.640 |
| Pi train | 23 | 0.500 | 0.500 | 17 | 0.824 | 0.176 | 10 | 0.400 | 0.600 |
| Meishan | 14 | 0.929 | 0.071 | 12 | 1.000 | 0.000 | 8 | 1.000 | 0.000 |
| Cinta Senese | 13 | 0.269 | 0.731 | 14 | 0.429 | 0.571 | 15 | 0.333 | 0.667 |
| Casertana | 28 | 0.589 | 0.411 | 27 | 0.296 | 0.704 | 28 | 0.554 | 0.446 |
| Calabrese | 14 | 0.571 | 0.429 | 16 | 0.688 | 0.312 | 16 | 0.594 | 0.406 |
| Nero Siciliano | 30 | 0.367 | 0.633 | 29 | 0.310 | 0.690 | 31 | 0.709 | 0.291 |

CA3 locus identified by sequencing does not create/disrupt a restriction site for any restriction enzyme, thus two new PCR primers were designed with the help of the dCAPS finder 2.0 software (Neff *et al.*, 2002) to analyse the identified point mutation by PCR-RFLP (forward 5'-TGAAAAGAG-TAAAACTGAAGTTGGG-3'; reverse 5'-TACGAAAGCATTTGGCCTTT-3') inserting an artificial restriction site for *HphI* by means of a mismatched base in the forward primer (underlined base in the forward primer; reverse primer was designed on sequence AJ604762). PCR conditions were as reported above apart from the annealing temperature that was set up at 61°C. Digestion of the resulting PCR fragment of 166 bp was performed for 5 µl of amplification product using 5 U of *HphI* (New England BioLabs Inc., Beverly, MA, USA) and the resulting products were resolved on 10% polyacrylamide/bisacrylamide 29:1 gels stained with ethidium bromide.

ATP1A2: A new PCR-RFLP assay for the *ATP1A2* mutation previously identified by SSCP and already characterised by sequencing (Russo *et al.*, 1999) was developed. As for

CA3, the C>G SNP of the *ATP1A2* gene does not create/disrupt a restriction site for any restriction enzyme. Thus with the help of the software described above, two PCR primers (forward: 5'-CCCTAAGGAAAATGGAAGAC-CGAATG-3'; reverse: 5'-GCCTGAT-GTTTGTCTTTTGTG-3') were designed on sequence Z98783 (Fontanesi *et al.*, 1999) to amplify a 62 bp product inserting an artificial restriction site for *HpyCH4V* by means of the forward mismatched primer (underlined base in the primer sequence). PCR conditions were as described above apart from the annealing temperature that was set up at 52°C. Five µl of amplification product was digested with 5 U of *HpyCH4V* (New England BioLabs Inc.) and the resulting fragments were visualized as described for *CA3*.

DECRI: PCR conditions and analysis of the polymorphism were as reported by Davoli *et al.* (2002a).

Allele frequencies at the *CA3* locus were studied in samples of unrelated pigs of Italian Large White (ILW), Italian Landrace, Italian Duroc (ID), Belgian Landrace, Hampshire, Pi train and Meishan breeds for a total of 222 pigs (Table 1). Furthermore,

allele frequencies at this locus were studied in animals of four Italian local pig breeds (Cinta Senese, Casertana, Calabrese and Nero Siciliano) that were chosen avoiding full-sibs (Table 1). For *ATP1A2* and *DECR1* allele frequencies were determined for the same local breeds (85 and 90 pigs, respectively), while for the other breeds allele frequencies were in part already reported by Russo *et al.* (1999) and Davoli *et al.* (2002a), respectively. For the animals used to investigate allele frequencies, phenotypic records or estimated breeding values (EBVs) were not available. These animals were different from those used to study the distribution of allele frequencies in extreme divergent groups of pigs (see below) and, henceforth this population will be named "random population".

Linkage mapping and radiation hybrid mapping

The DNA samples of pigs belonging to 6 three-generation families of the PiGMap Consortium (Archibald *et al.*, 1995) were genotyped at the *CA3* locus. The new PCR-RFLP protocol described for *ATP1A2* was used to genotype 4 three-generation families of the PiGMap Consortium increasing the number of informative meioses already obtained by Russo *et al.* (1999) who genotyped pigs of 7 other three-generation families of the PiGMap Consortium. The *DECR1* genotypes of the PiGMap reference families were already reported (Davoli *et al.*, 2002a). Two-point and multipoint procedures of the *CRI-MAP* package version 2.4 (Green *et al.*, 1990) were performed by merging the genotypes of these three loci with the genotypes of the loci already present in the PiGMap Consortium ResPig database (<http://www.resSpecies.org>). A two-point LOD score > 3 was used as a cut-off value for significant linkage. Multipoint sex-averaged map of SSC4 was constructed using options ALL, BUILD, CHROMPIC, FLIPS2-6.

The INRA-Minnesota 7000 rads radiation hybrid panel (Yerle *et al.*, 1998) IMPRH panel consisting of 118 rodent-porcine hybrid cell

lines was screened by means of PCR using the *ATP1A2* primers reported by Fontanesi *et al.* (1999), the *CA3* primers described for the SSCP analysis and the *DECR1* primers indicated by Davoli *et al.* (2002a). PCR conditions were as reported above except that for *ATP1A2* annealing temperature was set up at 57°C. No PCR fragment was obtained from the control rodent genomic DNA. The PCR reactions were visualized on 10% polyacrylamide/bis-acrylamide 29:1 or 2% agarose gels. The results of radiation hybrid PCR products were analysed with the IMPRH mapping tool developed by Milan *et al.* (2000) and accessible through the <http://imprh.toulouse.inra.fr/> web address. Multipoint locations of the three genes were obtained using minimum breakage criteria (Milan *et al.*, 2000).

Analysis of allele frequencies in extreme divergent groups of pigs

Estimated breeding values (EBVs) calculated by the National Association of Pig Breeders (Associazione Nazionale Allevatori Suini, ANAS; <http://www.anas.it>) were available for 3591 ILW pigs and 1225 ID pigs used for sib-testing during the period 1996-1999. A selection of animals for genotyping from these populations was performed in order to identify differences in allele frequencies between the two extreme tails of the distribution for several EBV in the considered genes. Among the ILW animals, 100 pigs with extreme divergent EBVs (50 with the highest and 50 with the lowest EBVs) were selected for each of the following traits: average daily gain (ADG; calculated from 30 kg to 155 kg of live weight with a *quasi ad libitum* nutritive level); weight of lean cuts (LC; that included neck and loin); back fat thickness (BFT; recorded at the level of *gluteus medius* muscle). For these three traits (ADG, LC and BFT) for which 300 extreme EBV of ILW pigs were considered, on the whole, 257 different animals were analysed as 43 pigs presented extreme values for more than one

Table 2. EBV means \pm SD for the two extreme groups (positive and negative) of chosen pigs for the four investigated traits.

| Traits | Positive groups of pigs | Negative groups of pigs |
|--------|-------------------------|-------------------------|
| ADG | +104.36 \pm 13.01 g | -60.34 \pm 13.12 g |
| LC | +5.95 \pm 0.49 kg | -3.74 \pm 0.53 kg |
| BFT | +10.69 \pm 3.19 mm | -11.40 \pm 1.07 mm |
| VIF | +2.17 \pm 0.34 | -2.35 \pm 0.27 |

ADG: Average Daily Gain; LC: Lean Cuts; BFT: Backfat thickness; VIF: Visible Intermuscular Fat.

trait. Among the available ID pigs, 100 animals with extreme divergent EBVs (50 with the highest and 50 with the lowest values) for visible intermuscular fat (VIF; expressed in units of standard deviation) were chosen. EBV means of the two extreme groups for each of the four investigated traits are reported in Table 2. DNA of the selected pigs was extracted from liophilized blood using a standard protocol (Sambrook *et al.*, 1989).

Then, these animals were genotyped at the *ATP1A2*, *CA3* and *DECRI* loci using the PCR-RFLP protocols described above. Fisher's exact test of significance (two tailed) of differences of allele frequency between the groups with positive and negative EBVs was calculated for each trait, considering in a first time all the animals and then both two-generation unrelated pigs and three-generation unrelated pigs among the chosen extreme animals. As we tested markers in a specific chromosome region known to harbour QTL, *P* nominal value of 0.05 was considered as the threshold for significance (Nagamine *et al.*, 2003).

Analysis of linkage disequilibrium (LD) between the three loci

Using the software 2LD (Zhao, 2002), LD (*D'* coefficient and its standard deviation; *D'* can range between 0, linkage equilibrium, to 1, complete LD; Lewontin, 1964; Hedrick,

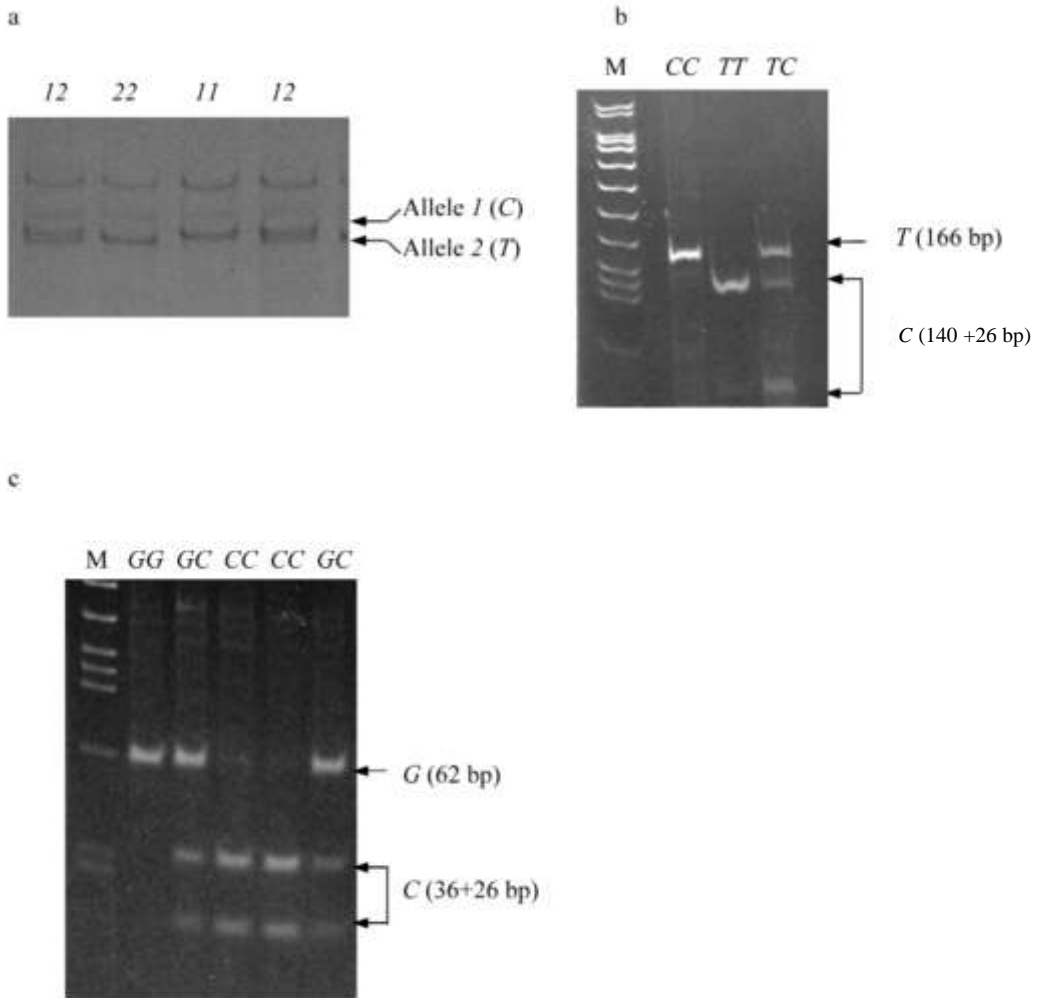
1987) was calculated between the three loci for the ID and ILW animals (100 and 257 pigs, respectively) used in the genotyping study of extreme divergent pigs described above. This parameter was calculated also in the sample of the animals used for the study of allele frequency in the "random population" described in Table 1. Moreover, the software allowed to calculate the probability to have *D'* value on the basis of a χ^2 analysis on observed haplotype frequencies and those expected under the hypothesis of linkage equilibrium.

Results and discussion

Identification and analysis of mutations

SSCP analysis made it possible to identify two alleles (indicated in this analysis as allele 1 and allele 2) in a 148 bp fragment of the 3'-UTR region of the porcine *CA3* gene (Figure 1, in a). Sequencing of two homozygous 11 and two homozygous 22 animals identified a SNP (C>T) at position 124 of the amplicon that is at position 150 of the sequence AJ301338. Allele 1 carries C while allele 2 carries T (EMBL accession no. AM040713 and AM040714, respectively). A PCR-RFLP protocol was set up to analyse this mutation. Allele 2 (*T*) is digested and the amplified fragment of 166 bp results in two fragments of 140 and 26 bp, while allele 1 (*C*) remains undigested (Figure 1, in b).

Figure 1. SSCP (a) at the *CA3* locus and analysis of this polymorphism by PCR-RFLP (b). Analysis of the *ATP1A2* SNP by PCR-RFLP (c). The genotypes are indicated at the top of each lane. M, DNA molecular weight VIII (Roche Diagnostics, Milano, Italy).



Frequencies of these alleles obtained analysing 297 pigs of different porcine breeds, including some cosmopolitan breeds, some Italian local breeds and the Chinese Meishan breed, are reported in Table 1.

To analyse the SNP identified at the *ATP1A2* locus that was previously genotyped by PCR-SSCP (Russo *et al.* 1999), a new PCR-RFLP protocol was developed.

Digestion of the 62 bp amplified fragment with *HpyCH4V* produced two fragments of 36 and 26 bp for allele *C* while allele *G* resulted unrestricted (Figure 1, in c). Allele frequencies at this locus for 11 different breeds are reported in Table 1. This Table also reports the allele frequencies in the same breeds at the *DECRI* locus, obtained analysing the mutation described by Davoli *et al.* (2002a).

Table 3. Allele frequencies and probability from Fisher's two-tailed exact test of equal frequency in positive vs negative groups for the polymorphisms at the *ATP1A2*, *CA3* and *DECR1* loci. Significant results ($P < 0.05$) are given in bold.

| Traits | Groups ¹ | N. of pigs ² | <i>ATP1A2</i> | | | <i>CA3</i> | | | <i>DECR1</i> | | |
|--------|---------------------|-------------------------|---------------|----------|----------|------------|----------|--------------|--------------|----------|--------------|
| | | | Allele C | Allele G | <i>P</i> | Allele C | Allele T | <i>P</i> | Allele G | Allele C | <i>P</i> |
| ADG | P | 50 | 0.245 | 0.755 | 0.999 | 0.090 | 0.910 | 0.096 | 0.400 | 0.600 | 0.256 |
| | N | 50 | 0.250 | 0.750 | | 0.180 | 0.820 | | 0.490 | 0.510 | |
| | P(u) | 18 | 0.250 | 0.750 | 0.802 | 0.111 | 0.889 | 0.413 | 0.444 | 0.556 | 0.840 |
| | N(u) | 37 | 0.216 | 0.784 | | 0.189 | 0.811 | | 0.473 | 0.527 | |
| LC | P | 50 | 0.230 | 0.770 | 0.517 | 0.160 | 0.840 | 0.019 | 0.650 | 0.350 | 0.085 |
| | N | 50 | 0.280 | 0.720 | | 0.310 | 0.690 | | 0.520 | 0.480 | |
| | P(u) | 31 | 0.177 | 0.823 | 0.121 | 0.177 | 0.823 | 0.026 | 0.629 | 0.371 | 0.310 |
| | N(u) | 42 | 0.298 | 0.702 | | 0.345 | 0.655 | | 0.536 | 0.464 | |
| | P(u)* | 16 | 0.250 | 0.750 | 0.809 | 0.250 | 0.750 | 0.174 | 0.594 | 0.406 | 0.828 |
| | N(u)* | 30 | 0.283 | 0.717 | | 0.400 | 0.600 | | 0.567 | 0.433 | |
| BFT | P | 50 | 0.260 | 0.740 | 0.999 | 0.250 | 0.750 | 0.739 | 0.450 | 0.550 | 0.119 |
| | N | 50 | 0.250 | 0.750 | | 0.220 | 0.780 | | 0.570 | 0.430 | |
| | P(u) | 35 | 0.286 | 0.714 | 0.705 | 0.271 | 0.729 | 0.321 | 0.443 | 0.557 | 0.130 |
| | N(u) | 36 | 0.250 | 0.750 | | 0.222 | 0.778 | | 0.583 | 0.417 | |
| VIF | P | 50 | 0.600 | 0.400 | 0.774 | 0.620 | 0.380 | 0.004 | 0.610 | 0.390 | 0.008 |
| | N | 50 | 0.570 | 0.430 | | 0.810 | 0.190 | | 0.790 | 0.210 | |
| | P(u) | 33 | 0.636 | 0.364 | 0.467 | 0.636 | 0.364 | 0.247 | 0.621 | 0.379 | 0.180 |
| | N(u) | 29 | 0.569 | 0.431 | | 0.741 | 0.259 | | 0.741 | 0.259 | |
| | P(u)* | 20 | 0.575 | 0.425 | 1.000 | 0.575 | 0.425 | 0.057 | 0.550 | 0.450 | 0.034 |
| | N(u)* | 21 | 0.571 | 0.429 | | 0.786 | 0.214 | | 0.786 | 0.214 | |

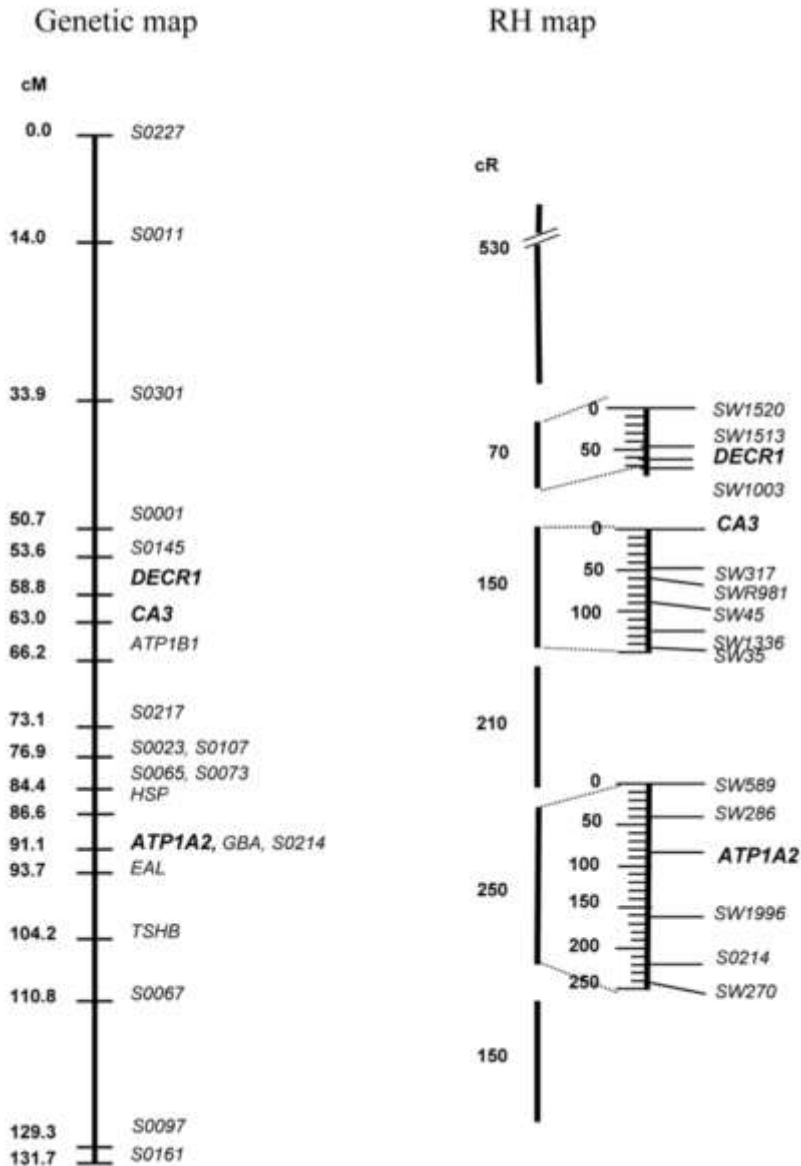
¹ P, positive EBV; N, negative EBV; P(u), positive EBV of two-generation unrelated pigs; N(u), negative EBV of two-generation unrelated pigs; P(u)*, positive EBV of three-generation unrelated pigs; N(u)*, negative EBV of three-generation unrelated pigs

² Number of pigs genotyped for each group

Construction of genetic and radiation hybrid maps of SSC4 containing the three investigated loci

To order the three analysed loci on the genetic map of SSC4, the polymorphisms identified in the three investigated genes were genotyped in the PiGMaP reference families. The number of informative meioses were 84, 86 and 62 for *ATP1A2*, *CA3* and *DECR1*, respectively. For the *CA3* locus (not yet analysed in the PiGMaP resource families), the two-point sex-specific procedure

revealed association with the following loci already mapped by Archibald *et al.* (1995) on SSC4: *S0001* ($\theta f = 0.00$, $\theta m = 0.15$, LOD = 5.30); *ATP1B1* ($\theta f = 0.00$, $\theta m = 0.00$, LOD = 5.12); *S0217* ($\theta f = 0.22$, $\theta m = 0.17$, LOD = 4.32); *S0214* ($\theta f = 0.07$, $\theta m = 0.07$, LOD = 3.71); *S0145* ($\theta f = 0.00$, $\theta m = 0.11$, LOD = 3.74). For the *ATP1A2* locus, for which additional linkage mapping data were obtained in the present study, the highest LOD score was observed with *S0073* ($\theta f = 0.07$, $\theta m = 0.00$, LOD = 14.64). For *DECR1* the linkage

Figure 2. Genetic and RH maps of SSC4 including *ATP1A2*, *CA3* and *DECRI*.

mapping results were already reported (Davoli *et al.* 2002a). Multipoint sex-averaged map of SSC4, constructed including these three loci all together, is shown in Fig. 2. *CA3* and *DECRI* resulted closely linked while *ATP1A2* was placed about 30 cM distal from the other two genes.

The results of the linkage mapping of *ATP1A2*, *CA3* and *DECRI* were confirmed by radiation hybrid (RH) mapping. The retention fraction for *ATP1A2* was 29%, for *CA3* was 28% and for *DECRI* was 50%. The microsatellites nearest to *ATP1A2*, *CA3* and *DECRI*, already placed on the RH map of

SSC4 (Hawken *et al.*, 1999) were *Sw286* (distance = 44 cR; LOD = 9.72), *Sw317* (distance = 54 cR; LOD = 7.58) and *Sw1003* (distance = 48 cR; LOD = 19.38), respectively. The multi-point RH map reporting the locations for the three genes, is shown in Fig. 2. The *ATP1A2* locus was placed between *Sw286* and *Sw1996*. This gene was assigned to the porcine RH map also by Moller *et al.* (2004). However, these authors positioned *ATP1A2* between markers *Sw589* and *Sw286*, thus there is no complete agreement with the position that we obtained. Further investigation using the pig 12,000 rad RH panel (IMNpRH2) (Yerle *et al.*, 2002) may refine the position of this gene and may solve the disagreement. *CA3* was assigned proximal in the RH group of *Sw317*. The result of the RH mapping of *DECRI* confirms the indication described by Clop *et al.* (2002) who reported that this locus was linked to *Sw1003*, even if no statistical support was given to their mapping. The position of these three genes is consistent with the conservation of syntenry and gene order between human chromosome (HSA) 8 (in which the human *DECRI* and *CA3* genes are localized) and HSA1 (in which the human *ATP1A2* is mapped) with parts of SSC4 as already described in detail by Moller *et al.* (2004).

The map position of the three loci was important to describe and discuss the results obtained in the next paragraph.

Genotyping results in extreme divergent groups of pigs and linkage disequilibrium data

Table 3 reports the allele frequencies at the three loci in the extreme divergent groups for the considered traits and the calculated P values. Both *CA3* and *DECRI* showed significant differences in allele frequencies for VIF and the former was significant also for LC. For *CA3*, allele *C* was more frequent in the negative group for LC ($P = 0.019$ for all the selected pigs; $P = 0.026$ for two generation unrelated pigs), and, on the other hand, it was the inverse for allele *T*.

For VIF, allele *C* resulted more frequent in the negative compared to the positive EBV group ($P = 0.004$) when all the animals were considered. This tendency was maintained in the two- ($P = 0.247$) and three-generation ($P = 0.057$) unrelated pig groups even if it was not significant. For the same trait, at the *DECRI* locus allele *G* increased its frequency in the group of pigs with negative EBV ($P = 0.008$, considering all animals; $P = 0.034$ for three-generation unrelated pigs). It is interesting to note that the two closely linked genes (*CA3* and *DECRI*) showed significant result for the same trait (VIF) analysed in the ID groups of pigs. For LC (analysed in the ILW) *CA3* showed significant differences but not *DECRI*, although the results for this gene approached the significance level ($P = 0.085$). The data for *ATP1A2* were never significant for any traits.

To evaluate these results in more detail, LD (D) was calculated between the three loci in the ID and ILW animals selected to compare the allele distribution in extreme EBV groups. A very high D' coefficient ($D' = 0.974$; s.d. = 0.025; $P < 0.0001$) resulted between *CA3* and *DECRI* in the ID animals while in the ILW animals the two loci showed a lower level of LD ($D' = 0.343$; s.d. = 0.089; $P = 0.0002$). It's interesting to point out that a high level of LD between *CA3* and *DECRI* was found for the same loci that showed significant values between extreme divergent Duroc pigs for VIF. This high level of LD may be the reason both loci showed significant results for VIF. The same high level of LD between *CA3* and *DECRI* was observed in the ID "random population" ($D' = 0.923$; s.d. = 0.072; $P < 0.0001$). A lower level of LD for these two loci in the ILW breed was also confirmed in the random population ($D' = 0.449$; s.d. = 0.237; $P = 0.079$). A significant LD between *CA3* and *DECRI* was confirmed, in general, also in the other breeds considered for the study of allele frequencies (data not shown). In the ID and ILW pigs, D' between *ATP1A2* and

CA3 was 0.178 (s.d. = 0.096; $P < 0.10$) and 0.058 (s.d. 0.057; $P = 0.289$), respectively. For *ATPIA2* and *DECR1*, D' was 0.217 (s.d. = 0.091; $P < 0.05$) and non significant ($D' = 0.007$; s.d. = 0.076; $P = 0.9214$) in ID and ILW, respectively. In general, considering the three loci, a higher LD in the ID population (average $D' = 0.178$; s.d. = 0.09; $P = 0.059$) was observed compared to the ILW pigs (average $D' = 0.058$; s.d. = 0.057; $P = 0.289$). An effect of the population and map distance between markers may be evident from the LD analysis of these three loci. These results confirm what was reported by Nsengimana *et al.* (2004), who, investigating the level of LD in commercial pig populations using microsatellite markers in a region of about 30 cM of SSC4 that includes *CA3* and *DECR1*, showed that the marker distance and the population were two factors that influenced the level of D' . A more complete analysis of LD in the ID breed may be important to evaluate the use of a LD approach to map QTL in this population.

Close to the *CA3* and *DECR1* genes, Gerbens *et al.* (1998) mapped the adipocyte fatty acid-binding locus (*A-FABP* or *FABP4* gene) and identified an association of this gene with intramuscular fat (IMF) content in a Dutch Duroc population. Considering the complete or almost complete conservation of gene order between SSC4 and human chromosome 8 (the human chromosome that contains *CA3*, *DECR1* and *FABP4*) (Moller *et al.*, 2004), it is possible to suppose that *FABP4* is very close and distal to *CA3*. As a matter of fact, *FABP4* maps at 82.6 Mb while *CA3* and *DECR1* are positioned at 86.5 and 91.1 Mb, respectively, in the physical map of HSA8 (Ensembl Human Genome Browser 2005). Furthermore, de Koning *et al.* (1999) using a cross between Meishan and Dutch Large White and Landrace lines indicated a suggestive QTL for IMF on SSC4 between *S0001* and *S0217*, the region that includes *DECR1*, *CA3* and *FABP4*. In a further analysis using the same cross population, Rattink *et al.* (2000) confirmed the

identification of a QTL for IMF in this region. The association between *FABP4* and IMF, the localization of a suggestive QTL for this trait on SSC4 between *S0001* and *S0217*, as well as the result obtained here for *CA3* and *DECR1* in the extreme divergent pigs for VIF, represent data obtained in different researches from various authors suggesting a significant effect on marbling/IMF of the SSC4 region where *CA3* and *DECR1* map.

It is relevant to note that in the present study the *ATPIA2* locus did not show any difference in allele frequencies for the investigated traits. This is unexpected considering that near the *ATPIA2* gene, Moller *et al.* (2004) located the *FAT1* locus, that according to several studies (i.e: Andersson *et al.*, 1994; Walling *et al.*, 2000), may have strong effects on several meat production and carcass traits. Moreover, the *ATPIA2* marker here investigated was localized in the correspondence of the QTL for some meat production and carcass traits (Cepica *et al.*, 2003). Furthermore, a study that used a logistic regression approach, suggested a putative association of *ATPIA2* on carcass traits in commercial pigs (Stefanon *et al.*, 2004). It is possible to suppose i) that the two alleles of *ATPIA2* are not in LD with the alleles at the *FAT1* locus in the analysed population ii) or that other QTL on SSC4 (namely in the region close to *CA3*) segregate in the ILW pigs. These hypotheses could be in agreement with Nagamine *et al.* (2003) who, studying five commercial pig populations, showed that segregation of QTL on SSC4 was evident only in two of them or to the fact that a few studies about the identification of QTL on SSC4 are not completely in agreement on the effects and position of these QTL (i.e: Walling *et al.*, 2000; Milan *et al.*, 2002; Cepica *et al.*, 2003; Mercadè *et al.*, 2005).

The effects of *DECR1* on meat quality and carcass traits were investigated by Amills *et al.* (2005) in a Landrace population. They analysed the same mutation that we identi-

fied and another missense mutation showing that only two haplotypes were present in their group of animals. Substantial differences among *DECRI* haplotypes with regard to growth and carcass traits were not observed and their results may be in agreement with the data we obtained in the ILW population. However, Stefanon *et al.* (2004) analysing the *DECRI* polymorphism in hybrid pigs, indicated that this gene may be involved in fat and protein deposition. This may suggest that further studies on *DECRI* are needed considering also the results on the distribution of its alleles in the extreme pigs for LC, that are close to the significance level.

Conclusions

SSC4 has been the objective of several studies because it harbours QTL with important effects on pig production traits. The SNP identified for *CA3* represents a new type I marker that could be used in QTL identification experiments and/or for fine mapping of already reported QTL. The construction of genetic and radiation hybrid maps of this chromosome including, all together, the three considered genes contributed to investigate the results obtained for the selective genotyping approach carried out to evaluate differences in allele frequencies between divergent groups of pigs for several production traits. The results that have been obtained are worth of further investigation, mainly for *CA3* and *DECRI* in the Duroc population, for which significant differences in the allele distribution in extreme groups for VIF were found. In this breed, of particular interest is also the high level of LD between these two markers. This information may be useful if population-wide linkage disequilibrium approaches are used to map QTL. The results of the present study can be considered to carry out association studies mainly to evaluate the effect of *CA3*.

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